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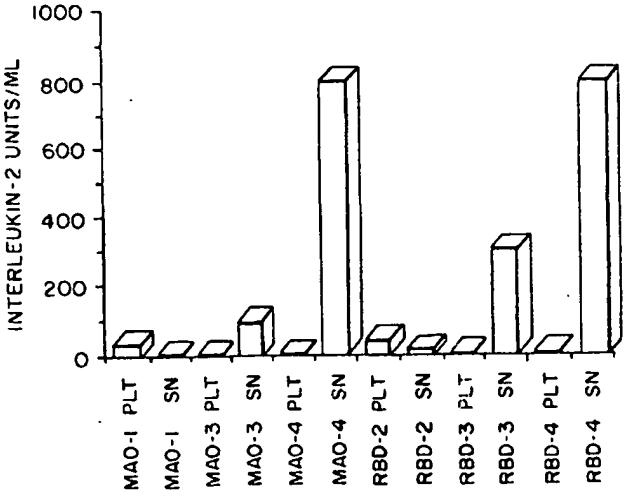

(57) Abstract

The present invention relates to genetically recombinant (genetically engineered) mycobacteria which express DNA of interest which has been incorporated into the mycobacteria and is expressed extrachromosomally (episomally or autonomously) in the recombinant mycobacteria under the control of a promoter. It particularly relates to recombinant *M. bovis*-BCG in which DNA of interest is expressed extrachromosomally under the control of a mycobacterial hsp promoter, such as hsp70 and hsp60. DNA of interest is defined herein as heterologous DNA (i.e., DNA from a source other than the mycobacterium into which it is introduced) and is all or a portion of a gene or genes encoding enzymes, cytokines, lymphokines and immunopotentiators.

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RECOMBINANT BCG VACCINES

Description

Background

Several viral and bacterial live recombinant vaccine vehicles are being developed to produce a new generation of vaccines against a broad spectrum of infectious diseases (Bloom, B. R., Nature 342:115-120 (1989)). The human tuberculosis vaccine Mycobacterium bovis bacillus Calmette-Guerin (M. bovis-BCG or BCG) (Calmette et al., Bull. Acad. Natl. Med. (Paris) 91:787-796 (1924)) has features that make it a particularly attractive live recombinant vaccine vehicle. BCG and other mycobacteria are highly effective adjuvants, and the immune response to mycobacteria has been studied extensively. With nearly 2 billion immunizations, BCG has a long record of safe use in man (Luelmo, F., Am. Rev. Respir. Dis. 125:70-72 (1982) and Lotte et al., Adv. Tuberc. Res. 21:107-193 (1984)). It is one of the few vaccines that can be given at birth, it engenders long-lived immune responses with only a single dose, and there is a worldwide distribution network with experience in BCG vaccination. Despite this widespread use of BCG, the mechanisms responsible for its diverse effects are still poorly understood.

To date, vaccines have been developed which, although effective in many instances in inducing immunity against a given pathogen, must be administered more than once and may be unable to provide protection, on a long-term basis, against a pathogen. In addition, in many cases (e.g., leprosy, malaria, etc.), an effective vaccine has yet to be developed.

While the complex immunologic stimulation engendered by BCG probably contributes to its efficacy as a vaccine, this complexity has also made it difficult to identify the mechanisms responsible for BCG's clinical effects. An understanding of BCG's mechanism of action might suggest ways to improve BCG's clinical efficacy.

Disclosure of the Invention

The present invention relates to genetically recombinant (genetically engineered) mycobacteria which express DNA of interest which has been incorporated into the mycobacteria and is expressed extrachromosomally (episomally or autonomously) in the recombinant mycobacteria under the control of a promoter. The mycobacterial heat shock protein (hsp) promoter, or stress protein promoter region (e.g., hsp70, hsp60) are particularly useful. One embodiment of the present invention particularly relates to recombinant M. bovis-BCG in which DNA of interest is expressed extrachromosomally under the control of a mycobacterial hsp promoter, such as hsp70 and hsp60. As used herein, the term DNA of interest refers to heterologous DNA (i.e., DNA from a source other than the mycobacterium into which it is introduced) which encodes a product (protein or polypeptide) which is an enzyme(s), a cytokine(s), a lymphokine(s) or an immunopotentiator(s); the DNA of interest can be all or a portion of a gene encoding the desired protein or polypeptide.

The present invention further relates to vaccines which are genetically recombinant mycobacteria, particularly recombinant BCG, which express DNA of interest extrachromosomally under the control of a mycobacterial hsp promoter and induce an immune response (e.g., T cell response) in mammals to whom they are administered.

Particularly, the present invention relates to a mycobacterial cytokine vaccine which is a recombinant mycobacterium which expresses and secretes a functional cytokine under the control of an appropriate promoter, such as a mycobacterial hsp promoter. As described herein, a mycobacterial cytokine vaccine which expresses and secretes a functional cytokine under the control of a mycobacterial hsp promoter has been shown to induce endogenous cytokine production, resulting in stimulation of T cells and macrophages. In addition, the recombinant mycobacterium has been shown to be a more potent stimulator of T cells and macrophages than the mycobacterium alone (wild type). In a specific embodiment, the recombinant mycobacterium which expresses and secretes a functional cytokine is recombinant BCG. The recombinant BCG has been shown to induce endogenous cytokine production to a greater extent than wild type BCG. The recombinant BCG expressing a cytokine offers a novel means of enhancing the host (e.g., human and other mammalian) immune response to BCG.

The recombinant mycobacteria can also be used as a vehicle for expression of lymphokines, immunopotentiators, enzymes, pharmacologic agents and antitumor agents. Recombinant mycobacteria can, for example, express protein(s) or polypeptide(s) which are growth inhibitors or are cytocidal for tumor cells (e.g., interferon α, β or interleukins 1-7, tumor necrosis factor (TNF) α or β and, thus, provide the basis for a new strategy for treating certain human cancers (e.g., bladder cancer, melanomas). The present invention also relates to methods of vaccinating a host with the recombinant mycobacterium to elicit protective immunity in the host. The recombinant vaccine can be used to produce humoral antibody immunity, cellular immunity (including helper and cytotoxic immunity) and/or mucosal or secretory immunity. In addition, the present invention relates to use of enzymes, cytokines,

lymphokines and immunopotentiators expressed by the recombinant cultivable mycobacterium as vaccines or as diagnostic reagents.

The vaccine of the subject invention has important advantages over presently-available vaccines. For example, mycobacteria have adjuvant properties among the best currently known. Thus, a recipient's immune system responds to the DNA of interest with great effectiveness. This is a particularly valuable aspect of the vaccine because it induces cell-mediated immunity. Thus, the vaccine will be especially useful in providing enhanced immunity in cases where cell-mediated immunity appears to be critical for cancer therapy or resistance to a pathogen. Second, the mycobacterium stimulates long-term memory or immunity. As a result, a single (one-time) inoculation can be used to produce long-term sensitization. Using the vaccine vehicle of the present invention, it is possible to prime long-lasting T cell memory, which stimulates secondary antibody response. Recombinant BCG of the present invention which express a cytokine, such as IL-2, are particularly useful because of their enhanced immunostimulatory properties (relative to nonrecombinant or wild type BCG). The present invention is, thus, useful to augment the immunostimulatory properties of BCG in immunization and cancer therapy. Any of a variety of cytokines can be expressed in recombinant mycobacteria, especially recombinant BCG, of the present invention.

BCG in particular has important advantages as a vaccine vehicle in that: 1) it is the only childhood vaccine currently given at birth; 2) in the past 40 years, it has had a very low incidence of adverse effects, when given as a vaccine against tuberculosis; and 3) it can be used repeatedly in an individual (e.g., in multiple forms).

A further advantage of BCG in particular, as well as mycobacteria in general, is the large size of its genome

(approximately 3 x 10^6 bp in length). Because the genome is large, it is able to accommodate a large amount of DNA from another source (i.e., DNA of interest) and, thus, can be used to make a multi-vaccine vehicle (e.g., one carrying DNA of interest encoding a cytokine and a lymphokine).

Brief Description of the Figures

Figure 1A is a schematic representation of the DNA maps of the relevant portions of the HSP60 promoter and polylinker (P) (SEQ ID No: 1) the Epitope tag sequence (T) (SEQ ID NO:2), and the BCG alpha antigen signal sequence (SS) (SEQ ID NO: 3) used in construction of IL-2 containing E. coli-BCG shuttle plasmids.

Figure 1B is a schematic illustration of IL-2 containing plasmid constructs with restriction sites; P=HSP60 promoter, T=Epitope tag, SS=BCG signal sequence; B=BamHI, E=EcoRI, H=HindIII, C=Cla I and S=Sal I.

Figure 2A is a western blot of BCG pellet (PLT) lysates and BCG culture supernatants (SN) from RBD recombinant clones detected with polyclonal rabbit anti-murine IL-2 antibody; Lane 1=recombinant murine IL-2, Lanes 2&3=MV261PLT and SN, Lanes 4&5=RBD-2 PLT and SN, Lanes 6&7=RBD-3 PLT and SN, Lanes 8&9=RBD-4 PLT and SN, respectively.

Figure 2B is the identical western blot in Figure 2a which was reprobed with murine monoclonal antibody 12 CA5 specific for the influenza epitope tag

Figure 3 is a graphic representation of expression of biologically active IL-2 in BCG pellet (PLT) lysates and BCG culture supernatants (SN) from rat (MAO) and mouse (RBD) recombinant BCG clones.

Figure 4A is a graphic representation of the time course of splenocyte interferon-γ production, in response to incubation alone (no Rx), or with IL-2 (IL-2), wild type BCG (BCG), or recombinant IL-2 secreting BCG (IL2-BCG).

2500 pg/ml of recombinant IL-2 was present at time zero in the IL-2 group; the amount of IL-2 present in the IL2-BCG group includes that produced by the recombinant BCG and that produced by splenocytes.

Figure 4B is a graphic representation of the time course of the splenocyte tumor necrosis factor production, in response to incubation alone (no Rx), or with IL-2 (IL-2), wild type BCG (BCG), or recombinant IL-2 secreting BCG (IL2-BCG). 2500 pg/ml of recombinant IL-2 was present at time zero in the IL-2 group; the amount of IL-2 present in the IL2-BCG group includes that produced by the recombinant BCG and that produced by splenocytes.

Figure 4C is a graphic representation of the time course of the splenocyte interleukin-6 production, in response to incubation alone (no Rx), or with IL-2 (IL-2), wild type BCG (BCG), or recombinant IL-2 secreting BCG (IL2-BCG). 2500 pg/ml of recombinant IL-2 was present at time zero in the IL-2 group; the amount of IL-2 present in the IL2-BCG group includes that produced by the recombinant BCG and that produced by splenocytes.

Figure 4D is a graphic representation of the time course of the total interleukin-2 production, in response to incubation alone (no Rx), or with IL-2 (IL-2), wild type BCG (BCG), or recombinant IL-2 secreting BCG (IL2-BCG). 2500 pg/ml of recombinant IL-2 was present at time zero in the IL-2 group; the amount of IL-2 present in the IL2-BCG group includes that produced by the recombinant BCG and that produced by splenocytes. (Delta-IL2, representing endogenous IL-2 produced by splenocytes was computed by subtracting the total IL-2 from the parallel experiment in which splenocytes were omitted.)

Figure 5A is a graphic representation of interferon-γ production by splenocytes derived from 3 mouse strains: C3H/HeN, C57BL/6 and BALB/c in response to wild type BCG (wtBCG).

Figure 5B is a graphic representation of interferon-γ production by splenocytes derived from 3 mouse strains: C3H/HeN, C57BL/6 and BALB/c in response to recombinant IL-2 secreting BCG (rBCG).

Figure 5C is a graphic representation of interferon-γ production by splenocytes derived from 3 mouse strains: C3H/HeN, C57BL/6 and BALB/c in response exogenous IL-2 (25 units=2500 pg) plus wtBCG.

Figure 6 is a western blot of the epitope tagged versions of cytokines in BCG; BCG-IL2 serves as a negative control and molecular weight markers are shown on the left.

Figure 7 is a schematic representation illustrating the possible mechanisms of secretion of cytokines from BCG; the small black bars represent the alpha-antigen signal sequence which is cleaved during export; arrows schematically represent the amount of protein secreted (See Figure 6).

Detailed Description of the Invention

Mycobacterium bovis-BCG (bacillus Calmette-Guerin) is an important clinical tool because of its immunostimulatory properties. Cell wall extracts of BCG have proven to have excellent immune adjuvant activity. Recently developed molecular genetic tools and methods for mycobacteria have provided the means to introduce foreign genes into BCG (Jacobs, W. R., Jr., et al., Nature 327:532-535 (1987), Snapper, S. B., et al., Proc. Natl. Acad. Sci. USA 85:6987-6991 (1988), Husson, R. N., et al., J. Bacteriol 172:519-524) and Martin, C., et al., Nature 345:739-743 (1990)). Live BCG is an effective and safe vaccine used worldwide to prevent tuberculosis.

The present invention relates to recombinant mycobacteria which express DNA of interest, which has been incorporated into the mycobacteria and is expressed extrachromosomally in the recombinant mycobacteria under

the control of a promoter. As used herein the term DNA of interest refers to heterologous DNA (i.e., DNA from a source other than the mycobacterium into which it was introduced) which encodes a product (protein or polypeptide) which is an enzyme(s), a cytokine(s), a lymphokine(s) and an immunopotentiator(s); the DNA of interest can be all or a portion of a gene encoding the desired protein or polypeptide. Recombinant mycobacteria of the present invention which express a cytokine or cytokines have enhanced immunostimulatory properties, when compared to the immunostimulatory properties of non-recombinant mycobacteria which do not express a cytokine or cytokines. The enhanced immunostimulatory properties of recombinant mycobacteria which express a cytokine or cytokines make the recombinant mycobacteria of the present particularly useful as vaccines.

As described herein, recombinant mycobacteria which express a cytokine have been produced and shown to have augmented or enhanced immunostimulatory properties, when compared with mycobacteria which do not express the cytokine. Cytokines are expressed and secreted in biologically active form in mycobacteria under the control of a mycobacterial promoter. The mycobacterial regulatory elements, particularly the hsp promoter, were selected because they are among the most powerful in bacteria (Neidhardt, F. C., et al., In Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology), F. C. Neidhardt, et al., eds., Washington, DC: American Society for Microbiology, 1654 pp (1987)) and because hsp (e.g.,hsp70) synthesis is induced to very high levels during phagocytosis of some bacteria by macrophage (Buchmeier et al., Science 248:730-732 (1990)). The hsp promoters also normally control the expression of proteins that are dominant antigens in the immune response to mycobacterial infection (Young, R. A., Annu. Rev. Immunol.

8:401-420 (1990), Lamb et al., Molec. Biol. and Med. 7:311-321 (1990), and Kaufmann, S. H. E., Immunol. Today 11:129-136 (1990)).

In Example 1, *M. bovis* BCG recombinants were constructed that produce and secrete the mammalian cytokine IL-2 in a biologically active form. Secretion of the active cytokine was accomplished through the combined use of the BCG hsp60 promoter and a secretion signal sequence derived from the BCG alpha-antigen. The BCG recombinants that secrete IL-2 have been shown to stimulate the production of specific lymphokines by mouse splenocyte cultures to a greater extent than wild type BCG stimulated their production. Thus, BCG recombinants that express IL-2 and other cytokines are a more potent stimulus of T cells and macrophages than the wild type BCG and can be used to modify the levels of specific cytokine production.

An in vitro prototype cytokine expression system for BCG is demonstrated in Example 1. As described, IL-2 encoding sequences are fused with the BCG alpha antigen signal sequence, resulting in expression and extracellular accumulation of biologically active IL-2. Additional evidence that the signal peptide was responsible for secretion was found in the Western blot analysis of the BCG recombinants. For each of the BCG recombinants that incorporated the signal sequence, the expressed IL-2 polypeptide appeared to accumulate in BCG cells both with and without the signal peptide; in contrast, the size of the single secreted form of IL-2 was consistent with that expected for IL-2 after the signal peptide has been cleaved. Matsuo et al.,previously demonstrated that HIV epitomes fused to the full length alpha antigen from *Mycobacterium kansasii* were secreted with the modified protein after signal peptide cleavage (Matsuo et al., Infect. Immun. 58:4049-4054 (1990)). However, there are no previous reports that the BCG alpha antigen signal peptide

itself could direct the extracellular secretion of a full length cloned protein from BCG.

The selection of the cytokine IL-2 as the first recombinant cytokine to be tested for secretion from BCG was based on the known central role of T cell mediated immune responses to BCG infection (Ratliff et al., J. Urol. 137:155-158 (1987)). An in vitro model of immune stimulation was developed using a mixed population of lymphocytes derived from spleen cells to determine if IL-2 secreting BCG would specifically affect a particular subset of T cells. A modest amount of γ-interferon production by naive splenocytes in response to BCG was found. IL-4 and IL-5 production, however, remained undetectable. This pattern of cytokine secretion by BCG is consistent with preferential T helper type one (TH-1) activation (Cherwinski et al., J. Exp. Med. 166:1229-1244 (1987)). A preferential stimulation of TH-1 cells has been described in splenocytes from C57BL/6 mice previously immunized with BCG or *Leishmania Major* (Chatelain et al., J. Immunol. 148:1182-1187 (1992)) and has been linked to major histocompatibility immune response genes (Huygen et al., Infect. Immun. 60:2880-2886 (1992) and Heinzel et al., J. Exp. Med. 169:59-72 (1989)).

The most dramatic results from the splenocyte assay were revealed for the IL-2 secreting BCG recombinant. Both IFN-γ and IL-2 production by splenocytes were increased approximately 7-8 fold over that produced by naive splenocytes treated with BCG alone. The effect on IFN-γ production was clearly synergistic, as neither BCG alone, IL-2 alone, nor the simple summation of their responses was able to generate such high levels. The production of IL-6 and TNF-α were also increased although to a much lesser extent. A remarkable finding, however, was the capacity of IL-2 secreting BCG to increase IFN-γ production from naive splenocytes to a level well beyond that found for

splenocytes treated with BCG alone. This effect was clearly related to the presence of IL-2 as it could be reproduced by the addition of exogenous IL-2 to wild type BCG. Furthermore, neutralizing antibody to IL-2 blocks this response. A synergistic increase in IFN-γ was shown to occur across 3 different mouse strains, supporting the concept that the local cytokine environment at the time of antigen presentation can significantly influence the direction and amplitude of the immune response. This is particularly significant for the BALB/c strain which characteristically is a poor IFN-γ producer (Heinzel et al., J. Exp. Med. 169:59-72 (1989)).

As described in Example 2, additional cytokines were tested for their ability to be secreted from recombinant mycobacteria. Using the vectors described in Example 1, BCG strains that secrete IL-6, GM-CSF, IL-4 and IFN-γ were constructed. Analysis of the recombinant mycobacteria described in Example 2 indicates several generalities may be evident when considering secretion of cytokines from BCG or other mycobacteria. First, the number of disulfide bonds in the final structure of the cytokine may be crucial. The data presented suggest that the fewer disulfide bonds in the mature protein, the more cytokine is secreted. For example, IL-2 is the best secreted cytokine and has one disulfide bond while IL-4 is the worst and has three disulfide bonds (Bazan, J.F., Science, 257:410-412 (1992); Smith L.J., et al., J. Mol. Biol., 244:899-904 (1992); Powers R., et al., Science, 256:1673-1677 (1992)). IL-6 and GM-CSF which have 2 disulfide bonds are intermediate, in terms of quantity produced, between IL-2 and IL-4 (Diederichs K., et al., Science, 254:1779-1782 (1991; Ealick, S.E., Science, 252:698-702 (1991)). Secondly, monomeric cytokines are likely to be more efficiently produced in an active form than oligomeric cytokines. Complex cytokines such as IL-12, which is a

heterodimeric molecule containing one intermolecular disulfide bond and multiple intramolecular disulfide bonds, is unlikely to be secreted in an active form. In contrast, IL-11, a molecule with similar biological activity to IL-6, has no disulfide bonds and is predicted to be monomeric, would be expected to be produced in large amounts from BCG.

These results suggest that the recombinant mycobacteria of the present invention has enhanced adjuvant and immunostimulatory properties above that found in wild type mycobacteria. The modifications described herein whereby BCG is engineered to provide a source of biologically active cytokines represents a novel means to enhance the host immune response to mycobacteria (e.g.,BCG) therapy and study its mechanism of action. In addition, other mycobacteria, such as Mycobacterium smegmatis, Mycobacterium avium, Mycobacterium phlei, Mycobacterium fortuitum, Mycobacterium lufu, Mycobacterium paratuberculosis, Mycobacterium habana, Mycobacterium scrofulaceiu, Mycobacterium intracellulare, Mycobacterium tuberculosis and any genetic variants thereof, can be used to practice the invention.

The adjuvant properties of BCG and its cell wall components have previously been exploited in experimental vaccines in animals and in man. For example, mixtures of BCG and specific schistosomal antigens have been used to successfully protect mice in a model of schistosomiasis (Pierce et al., Proc. Natl. Acad. Sci. USA 85:5678-5682 (1988)). An adjuvant/antigen mixture of muramyl dipeptide (MDP) and killed simian immunodeficiency virus (SIV) have provided partial protection against SIV infection in macaques (Desrosiers et al., Proc. Natl. Acad. Sci. USA 86:6353-6357 (1989) and Murphey-Corb et al., Science 246:1293-1297 (1989)); MDP is one of the components of mycobacterial cell walls that contributes to the adjuvant properties of BCG. Humans have been vaccinated with

mixtures of BCG and killed Mycobacterium leprae in large scale trials to assess the efficacy of this leprosy vaccine candidate (Bloom, B. R., J. Immunol. 137:i-x (1986)).

As demonstrated herein, recombinant mycobacteria, particularly BCG, can be used as a vaccine vehicle to express and secrete functional cytokines which induce endogenous cytokine production in cells. The recombinant mycobacteria secreting cytokines of the present invention can be used in vaccines for mycobacterial infections (e.g., tuberculosis or leprosy). For example, a tuberculosis vaccine can be developed in which M. tuberculosis is transformed with a plasmid of the present invention which contains a gene or genes encoding a cytokine to produce a rapid induction of an immune response against tuberculosis. In addition, the recombinant mycobacteria of the present invention can be used to enhance other vaccines as an adjuvant (e.g., added to a recombinant protein preparation or another BCG strain expressing another foreign antigen).

The ability to engineer BCG to express one or more genes encoding DNA of interest has several advantages over mixtures of mycobacterial adjuvant and pathogen antigens. Because the DNA of interest continues to be produced by BCG replicating in vivo, a BCG recombinant may provide a more long-lived immune response to the DNA of interest than that provided by the simple mixture of BCG DNA of interest. It may be more cost-effective to engineer BCG recombinants than to produce the mixture. Perhaps most importantly, the ease with which bacteria can be manipulated genetically makes it possible that features of the BCG vaccine vehicle can be tailored to maximize the desired immune responses.

In principle, a cytokine gene derived from any source can be introduced and expressed in mycobacteria, particularly BCG. The modified E. coli-BCG shuttle vectors described herein can be used to express and secrete a variety of cytokines. The expression of epitope tagged

cytokines from some of these vectors provides an additional assay for the presence of the cytokine. By being able to discriminate between the recombinant cytokine produced by BCG and the cytokine produced by mammalian cells, a more accurate picture of the mechanism of enhanced immunological stimulation can be obtained. In this embodiment, the DNA construct (e.g., E. coli-BCG shuttle plasmid) is comprised of the DNA of interest, a promoter and a secretion signal sequence wherein the 5' to 3' order is: the promoter, the secretion signal sequence and the DNA of interest, and the DNA of interest is under the control of the promoter. The DNA construct can additionally comprise an epitope tag for detecting the DNA of interest.

Components of the plasmid introduced into BCG or other mycobacterium (e.g., DNA of interest, hsp promoter and translational start site) can be obtained from sources in which they naturally occur or can be synthesized, using known techniques, to have substantially the same sequence as the naturally-occurring equivalent. For example, they can be produced by genetic engineering techniques (e.g., cloning), by the polymerase chain reaction or synthesized chemically.

It is also possible, using the method of the present invention, to construct a multipurpose or multifunctional vaccine (i.e, a single vaccine vehicle which contains and expresses DNA of interest which includes more than one gene). For example, it is possible to introduce into BCG a gene encoding IL-2 and a gene encoding IFN-γ. Administration of this multi-valent vaccine would result in a more potent immune response.

Vaccine vehicles of the present invention can be used to treat human cancers, such as bladder cancers or melanomas (e.g., by expressing growth inhibitors or cytocidal products). In this context, recombinant mycobacteria which contain and express cytokines (e.g.,

interferon α, β and/or γ, one or more interleukin (interleukins 1-7) and/or TNF α or β are particularly useful. In addition, the recombinant mycobacteria of the present invention can be used to reduce the dose of mycobacteria used in a clinical setting such as in the treatment for human bladder cancer or other cancers. For example, BCG has a proven beneficial effect in certain human bladder cancers but has variable side-effects. Using less BCG that is more immunogenic (e.g., BCG secreting a cytokine or combinations thereof) would be a major advance in this kind of therapy.

Further, the recombinant mycobacteria described herein can also be used to analyze of host-pathogen relationships. For example, recombinant mycobacteria of the present invention can be used for analysis of lymphocyte development in the context of mammalian systems of infectious disease and host-pathogen relationships by delivery of cytokines to the microenvironment of the infection sites.

Recombinant mycobacteria of the present invention can be administered by known methods. They can be administered by a variety of routes, such as intradermally or intravenously. They can be administered alone to produce a desired response, such as an immune response, or can be administered in combination with a killed or attenuated pathogen(s) against which an immune response is desired, in order to enhance or modify the resulting response.

The present invention will now be illustrated by the following examples, which are not to be considered limiting in any way.

Example 1 Recombinant BCG Secreting Functional Interleukin IL-2 Modulates Production of Splenocytes

Materials and Methods

Oligonucleotide primers, plasmid DNAs and bacterial strains.

Three sets of paired oligonucleotide primers were utilized in the polymerase chain reaction (PCR) with appropriate templates to produce insert DNAs with ends suitable for cloning in the plasmid pMV261. The oligonucleotide primers were:

for the rat IL-2 gene:
#1: GGCATGGCCAAGGGATCCGCACCCACTTCAAGCCCTGCA (SEQ ID NO: 4);
#2: CGGAATTCTTACTGAGTCATTGTTGAGATGAT (SEQ ID NO: 5);

for the mouse IL-2 gene:
#3: CAAGGGATCCGCACCCATTCAAGCCCTGCA (SEQ ID NO: 6);
#4: GCCGGAATTCTTACTGAGTCATTGTTGAGATGAT (SEQ ID NO: 7);

for the alpha antigen signal sequence:
#5: GCCATGCCACAGACGTGAGCCGAAAGATTCGA (SEQ ID NO: 8);
#6: GCCGGGATCCCGCGCCCGCGGTTGCCGCTCCGCC (SEQ ID NO: 9).

The rat and mouse IL-2 upstream primers #1 (SEQ ID NO: 4) and #3 (SEQ ID NO: 6), respectively, were constructed to anneal with the IL-2 coding regions starting at codon 21 thereby excluding their native signal peptide regions. The BCG alpha antigen downstream primer #4 (SEQ ID NO: 7), terminated at the sequence encoding the putative protease cleavage site ala-gly-ala (Terasaka et al, Complete nucleotide sequence of immunogenic protein MPB70 from *Mycobacterium bovis* BCG. FEMS Lett. 58:273-276 (1989)) (Figure 4A).

The rat IL-2 cDNA containing plasmid pRIL-2.8 was provided by A. McKnight and the mouse IL-2 cDNA plasmid pmut-1 was obtained through the ATCC (McKnight et al., Immunogen 30:145-147 (1989) and Yokota et al., Proc. Natl. Acad. Sci. USA 82:68-72 (1984)). The E. coli/BCG shuttle

plasmid pMV261 was kindly provided by C.K. Stover (Stover et al., Nature 351:456-460 (1991)). The influenza hemagglutinin epitope tag sequence (HA tag) is described in Kolodziej, P. A. and Young, R. A., Methods Enzymol. 194:508-519 (1991) and had been cloned in the Bgl II and Bam HI sites of pSP72. (Promega)

E. coli MBM 7070 was obtained from Michael Seidman. Mycobacterium bovis BCG (Pasteur) obtained from ATCC was grown in 7H9 media containing 10% albumin dextrose solution (Difco) and 0.05% tween 80 (Sigma). Genomic BCG DNA was isolated by protease K digestion and phenol/chloroform extraction.

Construction of IL-2 expression vectors and BCG IL-2 recombinant strains. A schematic representation of the plasmids constructed for this study is given in Figure 1B. The plasmid, pMAO-1, was constructed by placing the appropriate Bal I/Eco RI digested rat IL-2 PCR insert into the similarly restricted parental plasmid, pMV261 (Figure 1B). The plasmid, pMAO-2, was obtained by first cloning the Bam HI/Sal I insert from pMAO-1 into the HA tag containing plasmid (Figure 1A) and then placing the resulting BglII/EcoRI insert into the Bam HI/Eco RI site of pMV261. The plasmid, pMAO-3, was constructed by cloning the Bam I/Bal HI restricted PCR product encoding the alpha antigen signal sequence into the Bal I/Bam HI site of pMAO-1. The plasmid, pMAO-4, was produced by replacing the Bam HI/Eco RI insert of pMAO-3 with the Bgl II/Eco RI insert used in preparing pMAO-2. A similar set of mouse IL-2 containing plasmids, pRBD-1,2,3 and 4 was produced by replacing the Bam HI/Eco RI rat cDNA insert in each of the respective pMAO plasmids with the PCR derived Bam HI/Eco RI flanked mouse IL-2 cDNA fragment. All DNA manipulations followed previously described procedures in Maniatis et al., J.Molec. Cloning: a laboratory Manual, Cold Spring Harbor

Laboratories, Cold Spring, N.Y. (1982). E. coli MBM 7070 was electroporated with the IL-2 containing BCG/E. coli shuttle plasmids and selected on kanamycin (30 ug/ml) LB agar plates. The correct plasmid structures were confirmed on the basis of restriction analysis, DNA sequencing and production of functional IL-2 (see below). E. coli-derived plasmids were then used to transform BCG by electroporation according to published procedures (Snapper et al., Proc. Natl. Acad. Sci. USA 85:6987-6991 (1988)). BCG colony DNAs were individually tested by PCR for the presence of the IL-2 gene and colony lysates were assayed for expression of functional IL-2 (see below).

Detection of recombinant IL-2. The expression of recombinant IL-2 in BCG was examined by Western blot and a bioassay. Sonicated BCG lysates and BCG culture medium were electrophoresed on a 17-27% acrylamide gel (Daiichi) and transferred to nitrocellulose. After blocking the membrane with a 15% solution of powdered skim milk, the membrane was incubated overnight with the primary antibody, either rabbit anti-mouse IL-2 (Collaborative Research) or the mouse monoclonal anti-HA tag antibody 12CA5, at a concentration of 1 ug/ml (Wilson et al., Cell, 37:76 1984). Peroxidase labelled goat anti-rabbit or goat anti-mouse IgG antibodies (Pierce) were used with a chemiluminescent substrate (Amersham) for detection.

The presence of biologically active IL-2 in bacterial extracts or extracellular media was determined and quantified colorimetrically in a proliferation assay using the IL-2 dependent T cell line, CTLL-2 (Mosmann et al., J. Immun. Methods, 65:55-63 (1983)). Maximal signals generated in this assay were similar for either rat or mouse IL-2. E. coli and BCG lysates were obtained by sonication of washed bacterial cells in PBS followed by filtration through a 0.22 u filter and dialysis against

PBS. No IL-2 inhibitors were found when CTLL-2 cells were incubated with exogenous IL-2 in the presence of extracts prepared from bacteria transformed with the nonproducer plasmid, pMV261. To control for differing growth rates between BCG clones, log-phase BCG were washed and resuspended at an optical density of 0.5 at 600 nm (OD600) in fresh media. At the end of 48 hours, the OD600 was readjusted to 1.0 by diluting the BCG cells with fresh media. The amount of IL-2 in 1 ml (1.0 OD600 ~ 2-5 x 10^7 CFU) of cleared supernatant, or in the pellet derived from 1 ml of cells, was then assessed in the proliferation assay.

In vitro spleen cell assay for cytokine production.

Spleens were harvested from 8-12 week old C3H/HeN, C57BL/b or Balb/c mice (Charles River). After mechanical dispersion, the spleen cells were separated by Ficoll/hypaque centrifugation at 200 x g, washed, and placed into RPMI 1640 medium supplemented with HEPES (N-2-hydroxyethyliperazine-N'-2-ethanesulfonic acid), 10% heat inactivated fetal bovine serum, and 30 ug/ml of kanamycin. Splenocyte assays were performed with either 2 or 4 x 10^6 cells/well (1ml) in the presence or absence of exogenous murine recombinant IL-2 (Biosource), and either 2 x 10^6 CFU MV261 BCG (wild type BCG or wt BCG), or 2 x 10^6 CFU RBD-4 BCG. Duplicate supernatants were removed at 24 hours and 72 hours, centrifuged and frozen at -70°C until testing in ELISA assays. Equal spleen cell counts and viabilities were verified prior to final harvest by tryphan blue counting. Equal growth of wt BCG and RBD-4 during the 3-day experiment was verified by measurement of optical density at 600 nm for parallel wells containing supplemental 0.05% tween 80 to prevent bacterial clumping. Cytokine production by spleen cells was measured by commercial ELISA for murine cytokines, which were used according to the

manufacturer's instructions. Kits for the detection of murine IL-4,5,6, and TNF-α were purchased from Endogen. The IFN-γ ELISA was obtained from Gibco/BRL. IL-2 was assayed using a kit from Collaborative Research. To detect epitope tagged recombinant mouse IL-2, samples were incubated in wells precoated with rabbit anti-mouse IL-2, washed and reincubated with the murine monoclonal antibody 12CA5 at 1 ug/ml. Bound antibody was detected using peroxidase labelled goat anti-mouse IgG (Pierce et al., Proc. Natl. Acad. Sci. USA 85:5678-5682 (1988)).

Results

Construction of BCG recombinants producing IL-2

A variety of E. coli-BCG shuttle plasmids were constructed to permit production of IL-2 (Figure 1A and 1B). A set of plasmids was constructed in which the BCG HSP60 promoter drives the expression of mouse or rat IL-2 (pRBD-1 and pMAO-1). To permit differentiation of the BCG-produced recombinant IL-2 from IL-2 produced by mammalian cells in later experiments, a second set of plasmids was generated that incorporated an influenza hemagglutinin epitope coding sequence at the 5' end of the IL-2 coding sequence to produce an epitope-tagged IL-2 molecule (pRBD-2 and pMAO-2). To allow secretion of the recombinant IL-2 molecules, the secretion signal sequence of the mycobacterial alpha-antigen was added to the 5' end of the IL-2 coding sequence in a third set of plasmids (pRBD3 and pMAO-3). A fourth set of plasmids contained both the epitope tag and the secretion signal sequence upstream of IL-2 (pRBD- 4 and pMAO-4). All constructs containing the IL-2 gene were found to produce biologically active IL-2 in E. coli.

BCG cells were transformed with all of the recombinant plasmids. The BCG transformation efficiency for both the parental pMV261 and the constructs containing the alpha

antigen signal sequence were on the order of 10-100 times greater than those IL-2 constructs lacking the signal sequence. This was a uniform finding occurring in both mouse and rat IL-2 containing constructs and may be due to a selective disadvantage caused by the intracellular accumulation of this foreign protein.

IL-2 production and secretion by BCG transformants

The expression of IL-2 protein by representative BCG recombinants was assayed by probing Western blots with antibodies directed against IL-2 (Figure 2A) or against the influenza hemagglutinin epitope (Figure 2B). BCG recombinants that expressed IL-2 without a secretion signal sequence accumulated a single form of IL-2 intracellularly (Figure 2A, lane 5), but no IL-2 extracellularly (Figure 2A, lane 4). High and low molecular weight forms of IL-2 accumulated in BCG recombinants that expressed IL-2 linked to the secretion signal (Figure 2A, lanes 7-9); only the lower molecular weight form was found in the supernatant, consistent with the cleavage of the signal sequence during secretion (Figure 2A, lanes 6 and 8). The recombinant IL-2 proteins that contain the influenza hemagglutinin epitope tag can also be visualized with a monoclonal antibody specific for the tag (Figure 2B, lanes 5, 8 and 9).

The expression of IL-2 protein by representative BCG recombinants was also investigated using an IL-2-dependent proliferation assay (Figure 3). Most of the biologically active IL-2 produced by clones MAO-1 and RBD-2 was located in the pellet while most of the IL-2 product from clones MAO-3, MAO-4, RBD-3 and RBD-4 was found in the extracellular media. BCG clones expressing IL-2 linked to the alpha antigen signal peptide (MAO-3, MAO-4, RBD-3 and RBD-4) produced significantly more biologically active IL-2 than those clones without the signal peptide (MAO-1 and RBD-2). Both mouse and rat IL-2 BCG recombinants expressed

similar amounts of bioactive IL-2. The amounts of recombinant mouse IL-2 in pellets and in supernatants were also measured by an ELISA and similar results were obtained.

Stimulation of splenocyte cytokine production using BCG-IL-2 recombinants

To evaluate the immunostimulatory properties of IL-2 secreting BCG, the ability of BCG recombinants to alter the levels of cytokines IL-2, 4, 5, 6, TFN-α and γ-IFN produced by cultured murine spleen cells was investigated (Figure 4A-4D). Spleoncytes derived from C3H/HeN mice were incubated with either no BCG, 25 units/ml of IL-2, MV261 (wt)BCG or RBD-4 BCG. The levels of specific cytokines in the tissue culture media were measured by ELISA at 24 and 72 hours after the start of the experiment.

The data in Figure 4A-4D shows that no significant basal cytokine expression was detected from splenocytes in the absence of BCG or exogenous IL-2. In the IL-2 treated group, there was a modest elevation in IFN-γ production over the time course of the experiment, but no detectable increases in other cytokines. By contrast, splenocytes exposed to BCG produced significant amounts of IL-6, TFN-α and IFN-γ. However, the most significant cytokine production was observed with splenocytes exposed to BCG recombinants secreting IL-2. Substantially higher levels of IFN-γ were produced when spleen cells were exposed to recombinant BCG than when they were exposed to nonrecombinant BCG. Endogenous IL-2 production, as calculated by subtracting the total IL-2 in the absence of splenocytes from the total IL-2 in the presence of spleoncytes (Figure 4D, delta IL-2), also appeared to increase significantly. Finally, there was a more modest increase in TFN-α and IL-6 levels when spleen cells were exposed to recombinant BCG. We did not detect significant

amounts of either IL-4 or IL-5 in these splenocyte cultures (lower assay limit 100 ug/ml) under any of these experimental conditions.

There is a marked genetic variation in the amount of IFN-γ and IL-2 produced by splenocytes derived from mice infected by BCG (Huygen et al., Infect. Immun. 60:2880-2886 (1992)). For example, splenocytes from BCG-infected C57BL/6 mice produce high levels of IFN-γ and IL-2 while splenocytes from BCG-infected BALB/c mice produce low levels of these two cytokines after stimulation in vitro. To determine whether the enhanced immunostimulatory properties of IL-2 secreting BCG were strain independent, splenocytes were isolated from three different mouse strains, exposed to wild type or recombinant BCG, and the levels of specific cytokines in the tissue culture media were measured by ELISA at 24 and 72 hours. The results are shown in Figure 5A-5C. As in the previous experiment, there was very little IFN-γ production by C3H/HeN splenocytes stimulated with wtBCG (Figure 5A), but substantial levels were observed when the C3H/HeN splenocytes were stimulated with recombinant BCG (rBCG) producing IL-2 (Figure 5B). Enhanced stimulation was also observed with BALB/c and C57BL/6 splenocytes exposed to rBCG, although the levels of IFN-γ production were somewhat less with BALB/c and somewhat greater with C57BL/6. Similar results were obtained if exogenous IL-2 was added in the presence of wild type BCG (Figure 5C). There was no detectable IL-4 production in these splenocyte cultures. These results indicate that the enhanced immunostimulatory properties of IL-2 secreting BCG are not strain dependent.

Example 2 Recombinant BCG Secreting Cytokines

Materials and Methods

Construction of plasmids to secrete murine cytokines.

Murine cytokine cDNAs were cloned into the plasmids pRBD3 and pRBD4 described in detail in Example 1, which were used successfully to secrete murine and rat IL-2 from BCG. Recombinant BCG strains that secrete IL-6, GM-CSF, IL-4 and IFN-γ (all murine) were established. References for each cDNA used are as follows: GM-CSF (Gough, N.M., et al., EMBO J., 4:645-653 (1985)); IL-6 (Chiu, C.-P., et al., Proc. Natl. Acad, Sci. USA, 85:7099-7103 (1988)); IL-4 (Lee, F., et al., Proc. Natl. Acad. Sci. USA, 83:2061-2065 (1986)) and IFN-γ (Gray, P.W., et al., Proc. Natl. Acad. Sci USA, 80:5842-5846 (1983)). The expression vectors have a kanamycin resistance gene for selection in both BCG and *E. coli*, the HSP60 promoter upstream of the insert, a polylinker for insertion of the cDNA and DNA encoding the signal sequence derived from the BCG alpha antigen gene that targets the expressed protein for secretion through the mycobacterial cell membrane and wall (Matsuo *et al.*, Infec. Immun., 58:4049-4054 (1990)). The plasmid pRB4A also has the DNA encoding the 12CA5 epitope (Kolodziej and Young, Meth. Enzym., 194:508-519 (1991)) downstream of the signal sequence DNA for detection of the cytokine by immunoblotting. cDNAs were modified by PCR to remove the endogenous signal sequence encoding DNA with the following oligonucleotides: GM-CSF (PM32; CGCGGATCCGCACCCACCCGCTCA (SEQ. ID NO: 10) and PM33; GCGGAATTCTCATTTTTGGCTTGGTTT), (SEQ. ID NO: 11) IL-6 (PM34; CGCGGATCCTTCCCTACTTCACAA (SEQ. ID NO: 12) AND PM35; GCGGAATTCCTAGGTTTGCCGAGTAGA) (SEQ. ID NO: 13), IL-4 (PM36; CGCGGATCCAGGAGCCATATCCAC (SEQ. ID NO: 14) AND PM37; GCGGAATTCCTACGAGTAATCCATTTG) (SEQ. ID NO: 15) AND IFN-γ (PM38; CGCGGATCCCACGGCACAGTCATT (SEQ. ID NO: 16) AND PM39; GCGGAATTCTCAGCAGCGACTCCTTTT) (SEQ. ID NO: 17). Each oligonucleotide (listed 5′-3′) also contains the sequence for BamHI and EcoRI sites to be formed in the PCR product for cloning into the vectors.

Transformation and growth of mycobacteria

BCG (Pasteur strain) was transformed using previously published procedures (Aldovini and Young, Nature, 351:479-484, (1991); Aldovini et al., J. Bacteriol., 175:7282-7289 (1993). BCG were plated onto MH9 (Difco) plates with 20 μg/ml of kanamycin and transformants allowed to grow over the course of 4-8 weeks. Colonies were picked and expanded in 1-2 mls of MH9 broth with 20 μg/ml kanamycin. To find BCG clones that contained the plasmid of interest, BCG from liquid cultures was spotted onto nitrocellulose and allowed to air dry. The filters were then autoclaved for 2 minutes and then treated in the standard manner for colony hybridizations. The filters were then screened by hybridization with the relevant cytokine cDNA as a probe. Positive clones were further screened by examining the supernatant for the relevant cytokine by ELISA and bioassay. The highest producing cultures (expressed as pg/ml cytokine per OD600, see Table 1) were used in all future experiments.

Cytokine assays

Cultures of BCG that had been selected on kanamycin plates and were positive for the cytokine cDNA by hybridization as described above were screened for secretion of the cytokine into the culture supernatant. Supernatants were taken from cultures that had reached an approximate OD600 of 0.5-1.0 and initially filtered through a 0.22 μm filter and aliquoted and frozen at -70°C. Supernatants were screened by both ELISA and bioassay using standard procedures and cell lines. The ELISA kits were supplied by Endogen (IL-4, IL-6, GM-CSF and IFN-γ) or Genzyme (IL-2). The cell lines used for bioassays were CTLL and HT2 (IL-2), CT.4S (IL-4), FDC-P1 (GM-CSF), 7TD1 and KD83 (IL-6) and WEHI 279 and MB49.1 (IFN-γ). The sources of these cell lines were as follows: CTLL, CT4S

and HT2 from Drs. Andrew McKnight and Gary Singer (Brigham and Women's Hospital, Boston); FDC-P1 from Dr. Stephanie Watowich (Whitehead Institute); 7TD1 and WEHI 279 form the ATCC; KD83 and B16 lines producing the relevant cytokines from Dr. Glen Dranoff and Jason Salter (Whitehead Institute) and MB49.1 from Dr. Michael O'Donnell (Beth Israel Hospital, Boston). Various producer lines were used as standards in the bioassays and controls in ELISAs.

Western Blotting

BCG cultures were grown to an OD600 of approximately 2-3 in Middlebrook 7H9 media. Bacteria were isolated by centrifugation and lysed by sonication (Branson Ultrasonifier) in SDS sample buffer followed by boiling. The proteins in clarified supernatants were precipitated by the addition of 100% TCA to 5% followed by centrifugation and washing the pellet with cold acetone. The pellet was resuspended in SDS sample buffer. Samples were electrophoresed through 15% acrylamide gels followed by western blotting using standard procedures. The 12CA5 antibody (as ascites) was used at a final concentration of 1:1000 and detected with anti-mouse polyclonal antibodies coupled to alkaline phosphatase (Promega Biotec).

Results

Careful attention was paid to the known structures of murine cytokines in the assessment of what to attempt to secrete from BCG. We considered, given that little is known about secretion physiology in mycobacteria, that two important factors may plat a role in the successful secretion of murine cytokines. The first was the number of disulfide bonds in the cytokine. This is important because the cytoplasm of BCG, like other bacteria, is presumed to be a reducing environment for thiols (Derman et al., Science, 262:1744-1747 (1993)). Secretion of the cytokines

may depend upon the formation of correct disulfide bonds, presumably during and after the process of export from the bacterial cytoplasm. The second factor was the oligomerization state anticipated for each cytokine. In mammalian cells, an oligomeric cytokine, such as dimeric IFN-γ, would be formed in the endoplasmic reticulum and secretion apparatus where it would be concentrated prior to secretion. In the case of BCG, which lacks an endoplasmic reticulum, the cytokine will be exported without this concentration step. Thus, we anticipate that cytokines may undergo a significant dilution upon secretion and the concentration of *active* cytokine (if oligomerization is obligatory) would be reduced (see Figure 7). In this study, the latter only applies to IFN-γ as all the other cytokines are monomeric. The influence of these two factors will be elaborated upon below.

As described in Example 1, it is possible to have BCG secrete murine and rat IL-2. IL-2 has a single, essential disulfide bond and is monomeric. The other cytokines chosen for this study were GM-CSF, IL-6, IL-4 and IFN-γ. When the constructs were introduced into BCG and selected for with kanamycin, the number of colonies was generally reduced compared to the parental plasmid. Further, when these colonies were screened at random for secretion of cytokine, few positives were found. We adopted a screening procedure that first identified the presence of the plasmid in kan^r colonies followed by small scale screening for cytokine secretion. Using this procedure we were able to isolate cytokine secreting BCGs from large numbers of negative colonies. These data suggest that many of the cytokines may be toxic to BCG and the small numbers of positives isolated reflects the acquisition of secondary mutations in the mycobacteria that allow secretion of the cytokine of interest.

BCG strains secreting each cytokine were initially isolated based on specific ELISA detection. Levels of cytokine produced were also checked by western blotting using the 12CA5 antibody. Each cytokine cDNA had also been cloned into the vectors containing the coding region for the 12CA5 epitope (this is discussed in detail in the existing application). As shown in Figure 6 epitope-tagged cytokines were easily detected in the lysed BCG cell pellet, particularly for IL-2-tag, IL-4-tag and IFN-γ-tag. Levels of the IL-6-tag and GM-CSF-tag were more difficult to detect. Examination of the culture supernatants showed only IL-2-tag to be detectable. As will be shown below, this gives an indication of the amounts of cytokine inside the cell and secreted.

The assays used for each cytokine are detailed in the materials and methods and Table 1. Each cytokine was detected by cytokine-specific ELISA and the amount secreted is listed in Table 1. Note that the ELISAs are far more sensitive than western blotting for detection of each cytokine. It was then necessary to test if the cytokines secreted by BCG had biological activity. IL-2, IL-6 and GM-CSF were all easily detected by the relevant bioassay (Table 1). Epitope-tagged forms of each of these were also secreted and active in bioassays similar to the wild-type molecules. For IL-4, only small amounts were secreted into the culture medium that were below the limit of detection in the CT.4S assay. Eventually, an epitope-tagged strain was isolated that produced about ten times the amount compared to the wild-type IL-4 and was found to be active in the CT.4S bioassay although at the lower limit of the assay. For IFN-γ (only the tagged form was used here) large amounts were detected by a capture ELISA that detects both monomeric and dimeric IFN-γ. As dimerization is obligatory for function, we then tested whether the IFN-γ containing supernatants were active in two inhibition of

growth assays. Active IFN-γ was difficult to detect in either assay, suggesting that, although high levels of monomeric cytokine are secreted, the amount of dimer formed is low. The assays used here to detect IFN-γ activity are relatively insensitive and so we decided to make an assumption that the IFN-γ may be active *in vivo*, even in low amounts.

A summary diagram of what we envision is occurring during secretion is shown in Fig. 7. For IL-4 on the left of the diagram (this example applies to all monomeric cytokines listed in Table 1), small amounts of cytokine are secreted, consistent with the western blotting data, and disulphide bonds form and the molecules fold correctly outside the cell. For IFN-γ, shown on the right of Fig. 7, large amounts of cytokine are produced, a small fraction is secreted, but since a dilution occurs after export outside the cell, only a small percentage of IFN-γ will dimerize to the active form. This accounts for why a large amount of IFN-γ is detected by ELISA (which detects both monomeric and dimeric IFN-γ) but the amount detected in a bioassay low. We believe, however, the that BCG-IFN-γ construct will have activity *in vivo*, since only a minute amount of cytokine secreted at the sites of infection may be required to elicit a biological response.

TABLE 1

Analysis of BCG Secreting Cytokines

Clone	Transfection Efficiency	% Inserts	Disulphide Bonds	oligomerization?	Structure	pg/ml/ OD600	Bioassay	% Active
BCG-WT	100	--	--	monomer	--	--	--	--
BCG-IL-2	<5	~20	1	monomer	X-ray	>5000	wild-type#	100
BCG-GM-CSF	~50	100	2	monomer	NMR	1670	wild-type*	100
BCG-IL-6	~50	~50	2?	monomer	--	2000-2500	wild-type**	100
BCG-IL-4	<5	25	3	monomer	NMR,X-ray	129 (833)*	active$	?
BCG-IFN-γ	<5	~20	0	dimer	NMR	7140**	?=	?

* clearly positive in ELISA assays

** highly variable production from different clones

\# HT2 and CTLL assays

* FDC-P1 assay

** 7TD1, T1165 and KD83 assays

$ CT.4S assay

* WEHI-3 class II induction assay and WEHI-279 and MB-49.1 inhibition of growth assay.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A recombinant mycobacterium comprising DNA of interest which is expressed extrachromosomally under the control of a promoter, wherein the DNA of interest encodes a product selected from the group consisting of: enzymes, cytokines, lymphokines and immunopotentiators.

2. A recombinant mycobacterium of Claim 1 which is recombinant BCG and the promoter is a mycobacterial promoter.

3. A recombinant mycobacterium of Claim 2 wherein the mycobacterial promoter is a mycobacterial heat shock protein promoter.

4. A recombinant mycobacterium of Claim 3 wherein the mycobacterial heat shock protein promoter is the hsp70 promoter or the hsp60 promoter.

5. A recombinant mycobacterium of Claim 2 wherein the cytokine is selected from the group consisting of: interleukin-2, interleukin-4, interleukin-6, interferon-γ, interleukin-11 and GM-CSF.

6. A recombinant mycobacterium of Claim 1, which is selected from the group consisting of:
   a. Mycobacterium smegmatis,
   b. Mycobacterium bovis-BCG;
   c. Mycobacterium avium;
   d. Mycobacterium phlei;
   e. Mycobacterium fortuitum;
   f. Mycobacterium lufu;
   g. Mycobacterium paratuberculosis;

h. Mycobacterium habana;
i. Mycobacterium scrofulaceiu;
j. Mycobacterium intracellulare;
k. Mycobacterium tuberculosis; and
l. any genetic variants thereof.

7. A vaccine comprising the recombinant mycobacterium of Claim 1 and an appropriate carrier.

8. A BCG autonomous replicating plasmid vector comprising:
a) DNA of interest encoding a product selected from the group consisting of: enzymes, cytokines, lymphokines and immunopotentiators; and
b) DNA encoding a mycobacterial heat shock protein promoter and translational start site;
wherein the DNA of (a) is fused to the DNA of (b) in such a manner that expression of the DNA of (a) is under the control of the heat shock protein promoter and replaces the heat shock protein coding sequences.

9. A method of enhancing the immune response of a mammalian host against one or more pathogens, comprising administering to the host a recombinant mycobacterium, the recombinant mycobacterium having incorporated therein a plasmid comprising: DNA of interest encoding at least one cytokine fused to a mycobacterial heat shock protein promoter and translational start site in such a manner that expression of the DNA of interest is under the control of the mycobacterial heat shock protein promoter.

10. A method of Claim 9 wherein the recombinant mycobacterium is selected from the group consisting of:

a. Mycobacterium smegmatis,
b. Mycobacterium bovis-BCG;
c. Mycobacterium avium;
d. Mycobacterium phlei;
e. Mycobacterium fortuitum;
f. Mycobacterium lufu;
g. Mycobacterium paratuberculosis;
h. Mycobacterium habana;
i. Mycobacterium scrofulaceiu;
j. Mycobacterium intracellulare;
k. Mycobacterium tuberculosis; and
l. any genetic variants thereof.

11. A method of inducing production of a cytokine in a mammalian cell comprising contacting a mammalian cell with a recombinant mycobacterium, the recombinant mycobacterium having incorporated therein a plasmid comprising: DNA of interest encoding a cytokine and a mycobacterial heat shock gene promoter, wherein expression of the DNA of interest is under the control of the mycobacterial heat shock gene promoter and wherein the cytokine is expressed by the recombinant mycobacterium.

12. An E. coli-BCG shuttle plasmid comprising the following components:
a) DNA of interest encoding a product selected from the group consisting of: enzymes, cytokines, lymphokines and immunopotentiators;
b) a promoter; and
c) a secretion signal sequence
wherein the 5′ to 3′ order of the components is the promoter of (b), the secretion signal sequence of (c) and the DNA of interest of (a), and the expression of

the DNA of interest of (a) is under the control of the promoter of (b).

13. An E. coli-BCG shuttle plasmid of Claim 12 wherein the promoter is the mycobacterial heat shock protein 70 gene promoter or the mycobacterial heat shock protein 60 gene promoter; the secretion signal sequence is the BCG alpha antigen secretion signal sequence and which additionally comprises an epitope tag 5' of the DNA of interest.

14. A plasmid of Claim 13 wherein the epitope tag is influenza hemagglutinin.

15. A recombinant BCG with ability to provide an enhanced immunostimulatory effect in a mammal to which it is administered, said recombinant BCG comprising the following components:
a) DNA of interest encoding a product selected from the group consisting of: enzymes, cytokines, lymphokines and immunopotentiators;
b) a promoter; and
c) a secretion signal sequence
wherein the 5' to 3' order of the components is the promoter of (b), the secretion signal sequence of (c) and the DNA of interest of (a), and the expression of the DNA of interest of (a) is under the control of the promoter of (b).

16. A method of treating cancer in a mammal comprising administering recombinant BCG to a mammal, the recombinant BCG comprised of the following components:
a) DNA of interest encoding a product selected from the group consisting of: enzymes, cytokines, lymphokines and immunopotentiators;

b) a promoter; and

c) a secretion signal sequence

wherein the 5' to 3' order of the components is the promoter of (b), the secretion signal sequence of (c) and the DNA of interest of (a), and the expression of the DNA of interest of (a) is under the control of the promoter of (b).

17. A method of Claim 16 wherein the promoter is a mycobacterial heat shock gene promoter; the secretion signal sequence is the BCG alpha antigen secretion signal sequence and the which additionally comprises an epitope tag 5' of the DNA of interest.

18. A method of Claim 17 wherein the promoter is the mycobacterial heat shock protein 70 gene promoter or the mycobacterial heat shock protein 60 gene promoter and the epitope tag is influenza hemagglutinin.

19. A method of immunizing a mammalian host against M. tuberculosis infection comprising administering to the host a recombinant M. tuberculosis having incorporated therein a plasmid comprising: DNA of interest encoding at least one cytokine fused to a mycobacterial heat shock protein promoter and translational start site in such a manner that expression of the DNA of interest is under the control of the mycobacterial heat shock protein promoter whereby the presence of the plasmid in the mycobacteria results in a rapid induction of an immune response to M. tuberculosis.

20. A plasmid according to any one of Claims 8 and 12 wherein the promoter is the mycobacterial hsp70 or the hsp60 promoter.

21. A plasmid of Claim 20 wherein the cytokine is selected from the group consisting of: interleukin-2, interleukin-4, interleukin-6, interferon-γ, interleukin-11 and GM-CSF.

22. A method according to any one of Claims 9, 11, 16 and 19 wherein the cytokine is selected from the group consisting of: interleukin-2, interleukin-4, interleukin-6, interferon-γ, interleukin-11 and GM-CSF.

23. A method according to any one of Claims 9, 11 and 19 wherein the promoter is the hsp70 or the hsp60 promoter.

24. Use of a recombinant mycobacteria for inducing production of a cytokine in a mammalian cell comprising contacting a mammalian cell with a recombinant mycobacterium, the recombinant mycobacterium having incorporated therein a plasmid comprising: DNA of interest encoding a cytokine and a mycobacterial heat shock gene promoter, wherein expression of the DNA of interest is under the control of the mycobacterial heat shock gene promoter and wherein the cytokine is expressed by the recombinant mycobacterium.

HSP60 promoter and polylinker (P)

hsp60Pr-ATG GCC AAG ACA ATT GCG GAT CCA GCT GCA GAA TTC GAA GCT TAT CGA TGT CGA CGT
BalI BamHI EcoRI HindII ClaI SalI

Epitope tag sequence (T)

AatII
··· AGA TCT TCA CCA TAC GAC GTC CCA GAC TAC GCT GGA TCC TCT AGA GTC GAC —
BglII Influenza HA epitope tag 12CA5 BamHI XbaI SalI

BCG alpha antigen signal sequence (SS)

BalI
A TG GCC ACA GAC GTG AGC CGA AAG ATT CGA GCT TGG GGA CGC CGA TTG ATG ATC
GGC ACG GCA GCG GCT GTA GTC CTT CCG GGC CTG GTG GGG CTT GCC
GGC GGA GCG GCA ACC GCG GGC GCG GGATCC
BamHI

FIG. 1A

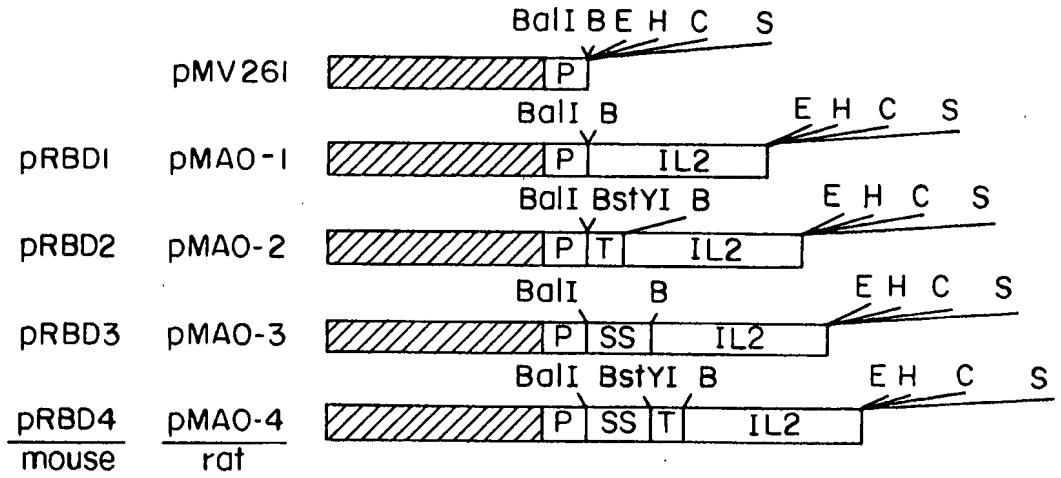


FIG. 1B

FIG. 2A

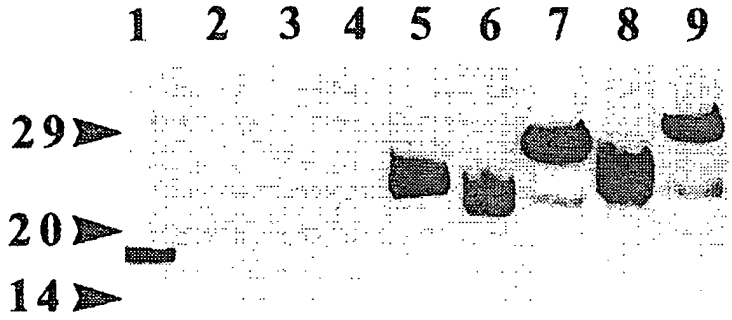
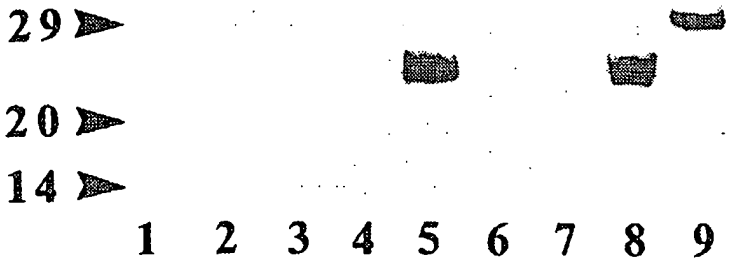


FIG. 2B



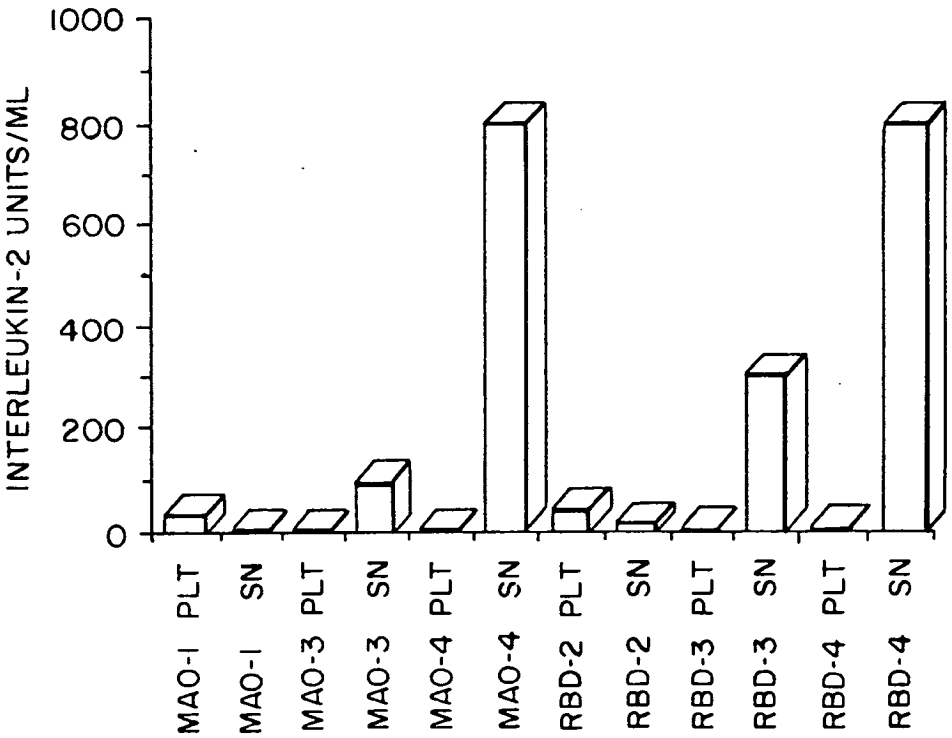

INTERLEUKIN-2 UNITS/ML
1000
800
600
400
200
0
MAO-1 PLT
MAO-1 SN
MAO-3 PLT
MAO-3 SN
MAO-4 PLT
MAO-4 SN
RBD-2 PLT
RBD-2 SN
RBD-3 PLT
RBD-3 SN
RBD-4 PLT
RBD-4 SN

FIG. 3

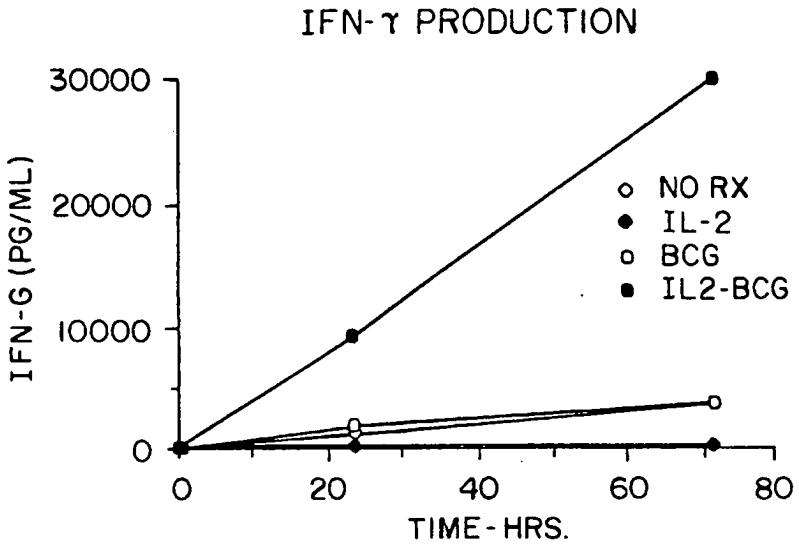

IFN-γ PRODUCTION
IFN-G (PG/ML)
30000
20000
10000
0
0
20
40
60
80
TIME-HRS.
NO RX
IL-2
BCG
IL2-BCG

FIG. 4A

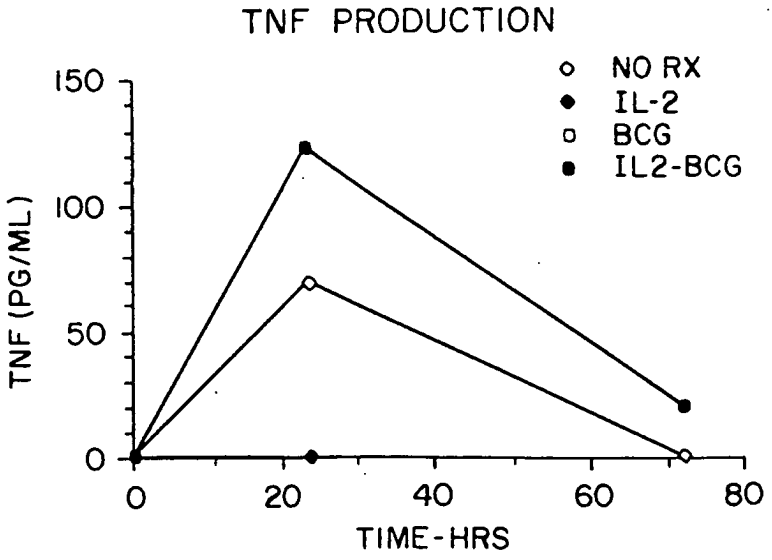

TNF PRODUCTION
TNF (PG/ML)
150
100
50
0
0
20
40
60
80
TIME-HRS
NO RX
IL-2
BCG
IL2-BCG

FIG. 4B

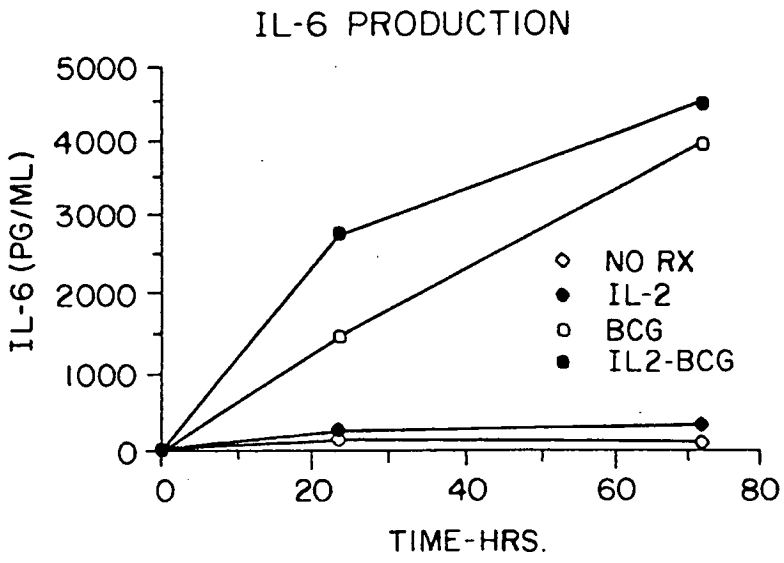

IL-6 PRODUCTION
IL-6 (PG/ML)
5000
4000
3000
2000
1000
0
0
20
40
60
80
TIME-HRS.
NO RX
IL-2
BCG
IL2-BCG

FIG. 4C

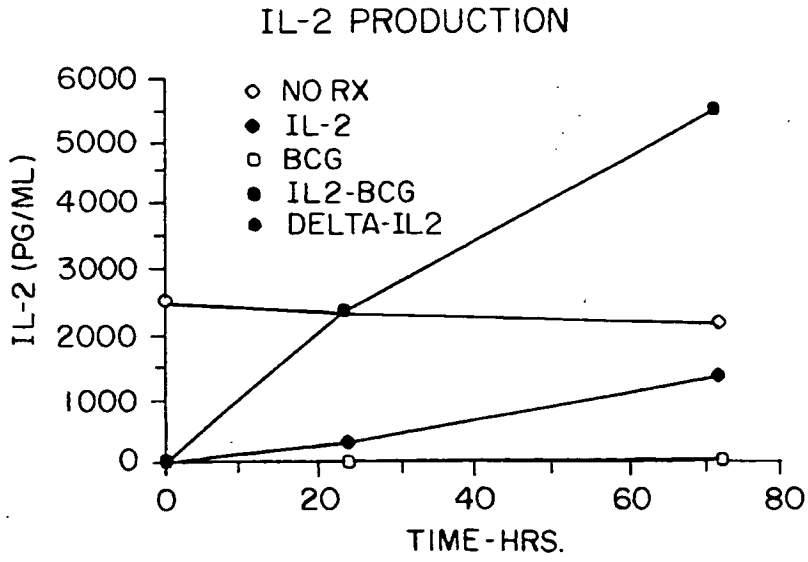

IL-2 PRODUCTION
IL-2 (PG/ML)
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
TIME-HRS.
NO RX
IL-2
BCG
IL2-BCG
DELTA-IL2

FIG. 4D

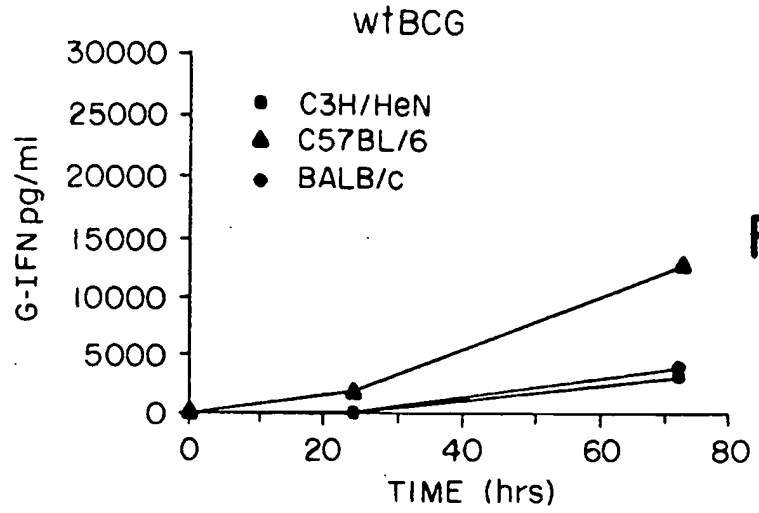


FIG. 5A

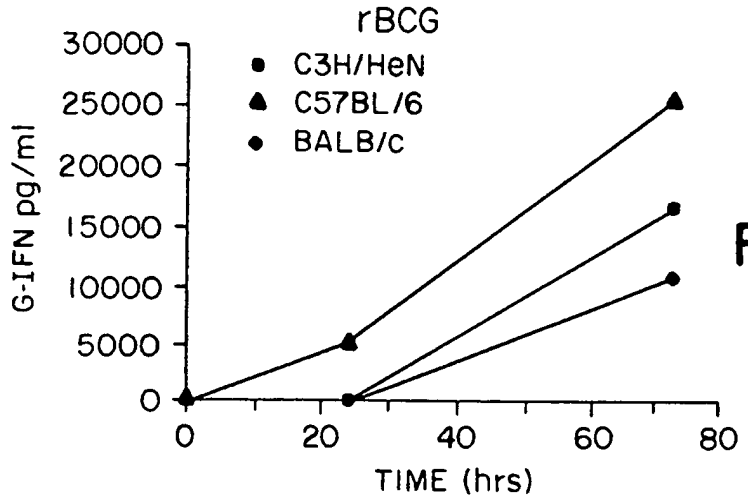


FIG. 5B

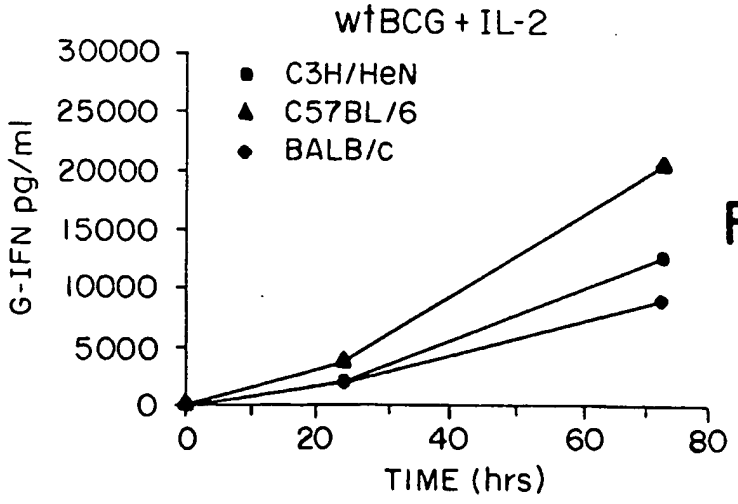


FIG. 5C

BCG-IL-2
BCG-IL-2-Tag
BCG-GM-CSF-Tag
BCG-IL-6-Tag
BCG-IL-4-Tag
BCG-IFN-γ-Tag
BCG-IL-2
BCG-IL-2-Tag
BCG-GM-CSF-Tag
BCG-IL-6-Tag
BCG-IL-4-Tag
BCG-IFN-γ-Tag
BCG Pellets
BCG Culture Supernatants
30
14.3

FIG. 6

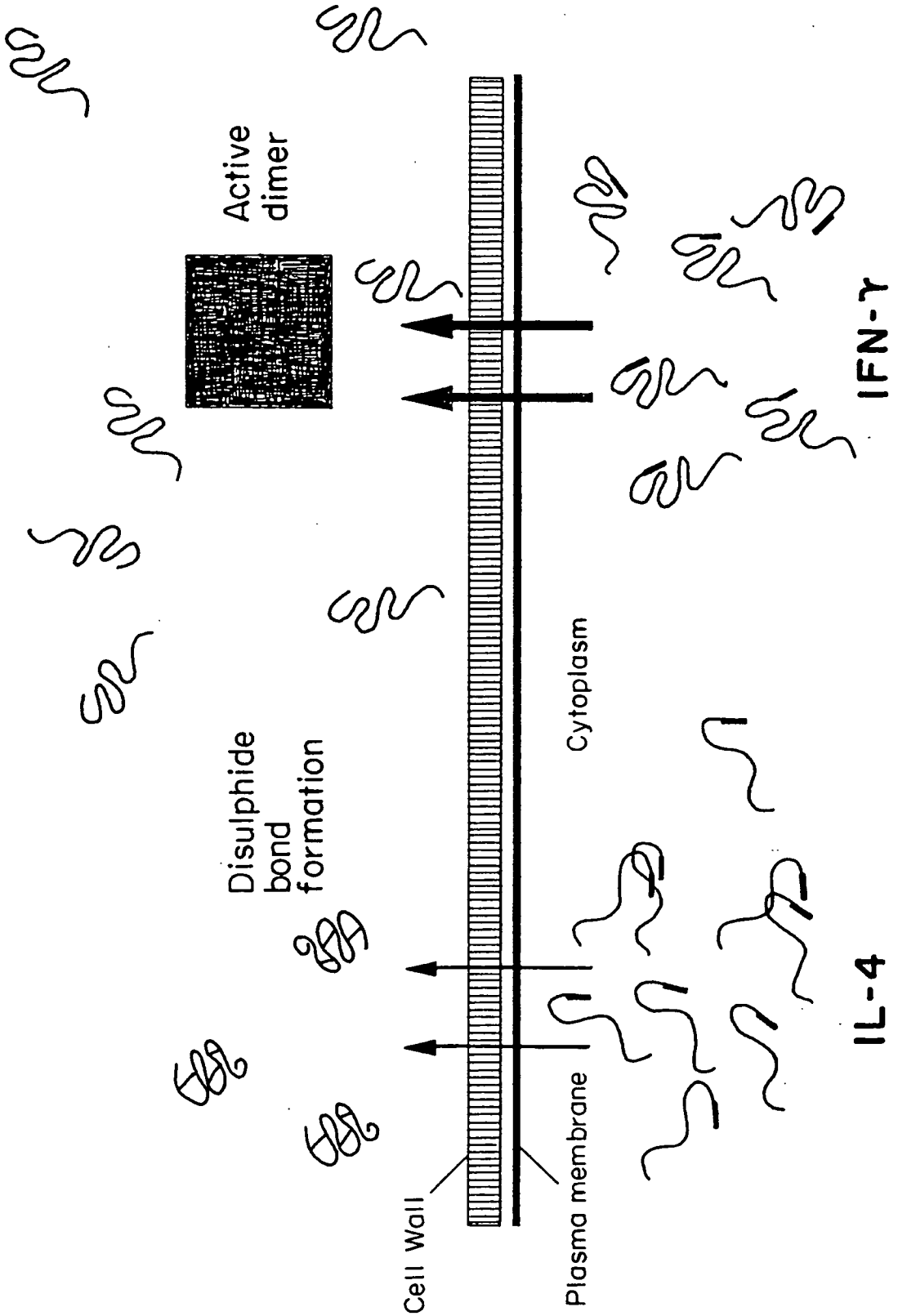

Active dimer
IFN-γ
Cytoplasm
Disulphide bond formation
IL-4
Cell Wall
Plasma membrane

FIG. 7